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Freezing Resistance of a *Trichinella spiralis nativa* Isolate from a Gray Wolf, *Canis lupus,* in Montana, with Observations on Genetic and Biological Characteristics of the Biotype

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ABSTRACT: A *Trichinella spiralis* isolate obtained from a gray wolf (*Canis lupus*) in northwestern Montana in 1987 was evaluated for infectivity and resistance to freezing and its DNA restriction fragment length polymorphisms (RFLP's) compared to that from other sylvatic and domestic isolates. Infectivity indices in albino mice were low (≤2.6) and correlated well with mouse infectivity data from other sylvatic isolates. Larvae did not survive freezing after storage at −20 to −30°C in wolf musculature for 1- and 2-mo periods. Analysis of DNA extracted from the wolf isolate by RFLP's and DNA dot blots using a *Trichinella spiralis spiralis*-specific probe indicated that this isolate belongs to the *Trichinella spiralis nativa* group. Inability of the Montana wolf isolate to survive subfreezing temperatures contrasts with published data indicating long-term survival (>18 mo at −10°C) of a Canadian wolf isolate. This disparity in cold-hardiness of *T. spiralis* isolates from wolves in neighboring zoogeographic regions as well as variations in RFLP banding patterns between the Montana wolf isolate and other freeze-resistant isolates (e.g., polar bear) indicates the coexistence of dissimilar sylvatic strains at higher latitudes where Arctic or freeze-resistant biotypes predominate.

KEY WORDS: *Trichinella spiralis,* wolf strain freeze resistance, infectivity in deer mice, DNA polymorphisms.

Selected biological features such as host affinity, infectivity, and fecundity in laboratory rodents, and resistance to freezing have been proposed as working criteria for differentiating *Trichinella spiralis* subspecies (Dick and Chadee, 1981; Leiby et al., 1985; Worley et al., 1985). Recently, isozyme analysis (Pozio et al., 1989), DNA probes (Klassen et al., 1986b; Dame et al., 1987), and characteristic banding patterns of enzyme-digested parasite DNA separated on agarose gels (Curran et al., 1985; Chambers et al., 1986; Klassen et al., 1986a; Zarlenga and Murrell, 1989) have also been used to distinguish *T. spiralis* isolates. Furthermore, some physiological and ecological characteristics appear to be relatively constant within subspecies and hence may have value for differentiating *Trichinella spiralis spiralis* and *Trichinella spiralis nativa* (Belosevic and Dick, 1980; Smith, 1983). Experimental data derived from a wolf isolate collected in northwestern Montana demonstrated that isolates of *T. s. nativa* from the same host species and geographic region may have contrasting biological characteristics such as resistance to freezing.

Materials and Methods

Trichinella larvae obtained by peptic digestion of skeletal musculature from an adult male gray wolf (*Canis lupus*) killed on the Blackfeet Indian Reservation adjoining Glacier National Park at approximately 48°30′N latitude, 113°W longitude in 1987 were evaluated for resistance to freezing as described previously (Worley et al., 1986). Parasite infectivity was verified by passages in both deer mice (*Peromyscus maniculatus*) and CD-1 strain albino mice (*Mus musculus*). Genomic DNA extracted from the Montana wolf isolate by proteinase-K SDS digestion (Dame and McCutchan, 1983) was compared by dot blot and RFLP analysis to DNA isolated from several pig and *T. s. nativa* isolates (see Dame et al., 1987, for complete listing and source of parasite isolates).

Dot blots were prepared by diluting 0.5 μg of each genomic DNA to 400 μl in 10 mM Tris, pH 7.6, 1 mM EDTA, and heating the solution in a boiling waterbath for 10 min. The solutions were adjusted to 4× SSC (1× SSC is 0.15 M sodium chloride, 0.015 M sodium citrate, pH 7.0) and vacuum-filtered through a nitrocellulose membrane, which was subsequently baked at 80°C for 2 hr. The filters were prehybridized at 65°C for 6 hr in hybridization buffer. Dot blots were then screened overnight with a ^{32}P-labeled *T. s. spiralis*-specific probe (pBP-2) (Dame et al., 1987) prepared by nick translation (Rigby et al., 1977) to determine the relationship of the Montana wolf isolate to the pig isolate. Filters were washed in 0.2× SSC, 0.1% SDS at 50°C, air dried, and then autoradiographed.

Southern blot analysis was used to compare restriction enzyme-digested *T. spiralis* DNA from Montana wolf to DNA isolated from *T. s. spiralis* collected from pig and various sylvatic hosts. DNA samples (2 μg) were digested to completion for 2 hr with Dra I (10 units of enzyme/μg DNA), electrophoresed through a 0.8% agarose gel, and then blotted to a Nytran mem-

Table 1. Infectivity in deer mice (*Peromyscus maniculatus*) of a wolf isolate of *Trichinella spiralis nativa* before and after freezing in wolf muscle.

Time frozen (days)	No. mice inoculated	Inoculum* (larvae/mouse)	Mice infected/total no. inoculated	Larvae/g bodyweight
0	3	100	3/3	83, 89, 283
30	3	100	0/3	—
60	3	100	0/3	—

* Given orally in saline suspension.

Table 2. Comparative infectivity of *Trichinella spiralis* isolates from domestic pig and gray wolf in albino mice.

Host	No. mice inoculated	Infectivity index*
Pig	7	24.25
Wolf	10	2.53

* Index: ratio of larvae inoculated to total muscle larvae recovered 44 days postinoculation.

brane according to Southern (1975). Membrane filters were baked and prehybridized as described above. Blots were screened with [γ^{32}P]ATP-kinased total RNA (Zarlenga and Gamble, 1987) then hybridized and washed as indicated for dot blot analysis.

Results and Discussion

Digestion of a 25-g sample of wolf biceps brachii muscle revealed a larval concentration of 26.3 larvae/g of tissue. Parasite infectivity was verified by successful passages in deer mice (Table 1); however, infectivities in albino mice were significantly lower than *T. s. spiralis,* suggesting that the isolates from the wolf and pig were not the same (Table 2).

Because it had been demonstrated elsewhere (Dame et al., 1987; Murrell et al., 1987) that *T. s. spiralis* is capable of infecting sylvatic hosts, it was necessary to determine whether the gray wolf isolate was of the pig type (*T. s. spiralis*) or more closely related to other sylvatic isolates (*T. s. nativa*). Dot blots screened with a ^{32}P-labeled probe (pBP-2), specific for *T. s. spiralis,* verified that the Montana wolf isolate was not of the pig biotype by its failure to hybridize to the pBP-2 probe (Fig. 1). Furthermore, Southern blot analysis comparing restriction enzyme-digested DNA from pig and sylvatic *T. spiralis* isolates indicated similarities between the wolf isolate and *T. s. nativa* (Fig. 2, lanes 2–5) and significant differences in the major rDNA bands from the pig type (Fig. 2, lane 1).

No viable *Trichinella* larvae were retrieved from wolf leg musculature stored at −20 to −30°C for 1- and 2-mo periods as indicated by failure to induce infections in laboratory-reared deer

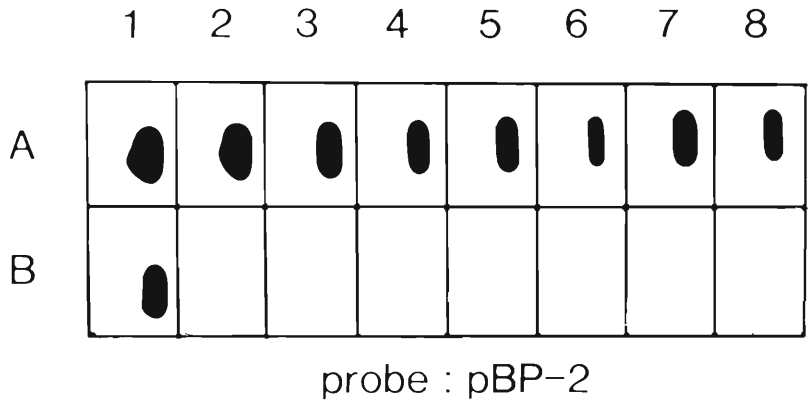

Figure 1. Dot blot analysis of *T. spiralis* isolates using pBP-2 probe. DNA samples (0.5 μg) were denatured by boiling and then vacuum-filtered through a nitrocellulose membrane. Blots were baked at 80°C for 2 hr and then hybridized to the ^{32}P-labeled pBP-2 DNA probe. A: 1, Beltsville pig (*Sus scrofa*); 2, Maine pig (*S. scrofa*); 3, Scott pig (*S. scrofa*); 4, Thai pig (*S. scrofa*); 5, bobcat (*Felis rufus*); 6, polar bear—4 (*Ursus maritimus*); 7, raccoon—1 (*Procyon lotor*); 8, UPB-6 (*Ursus americanus*). B: 1, UPB-8 (*U. americanus*); 2, Montana wolf (*Canis lupus*); 3, grizzly—1 (*Ursus arctos*); 4, polar bear—1 (*U. maritimus*); 5, Pennsylvania fox (*Urocyon cinereoargenteus*); 6, UPB-3 (*U. americanus*); 7, UPB-11 (*U. americanus*).

mice. The absence of freezing resistance in muscle larvae from the Montana wolf, which was a member of a pack believed to have originated farther north along the Canadian Rockies in Alberta (Ream and Harris, 1986), contrasts with long-term survival (18 mo at −10°C) reported by Dies (1980) for *Trichinella* larvae in skeletal muscle of a wolf collected near Fort McMurray in northwestern Alberta. Larval viability of the Canadian isolate was also confirmed by mouse inoculation. Because both isolates were derived from wolves inhabiting adjoining biotic provinces differing more in physiographic features than climate (Dice, 1943), and because each had been shown to be true sylvatic isolates by DNA hybridization studies and freeze resistance, an obvious explanation for the disparity in cold hardiness of the 2 isolates is lacking. RFLP comparison of the Montana wolf isolate (Fig. 2, lane 2) with a previously determined freeze-resistant isolate, PB-1 (Fig. 2, lane 4), reveals some variation in banding patterns, i.e., absence of the 1.8-kb, 2.6-kb, and 4.1-kb bands in the freeze-resistant strain. Whether these band differences are indicative of the cold-hardy biotype or are attributable to normal variation within the various sylvatic isolates is not yet known.

Previous studies have clearly demonstrated the existence of freeze-resistant isolates of *T. spiralis* in certain host species (e.g., grizzly bear and wolverine) at latitudes comparable to that where the Montana wolf isolate originated (Worley et al., 1986). As neither host muscle type nor relative depth of larvae within infected tissue appear to alter the ability of trichinae to survive subfreezing temperatures (Chadee and Dick, 1982), factors other than host range, geographic origin, and tissue distribution of larvae must be considered in evaluating the existence of so-called Arctic or cold-hardy *T. spiralis* isolates occurring at higher latitudes in North America. The regional variability in parasite subpopulations from the same host inhabiting different sylvatic ecosystems may better be studied by developing DNA probes for the freeze-resistant genotype.

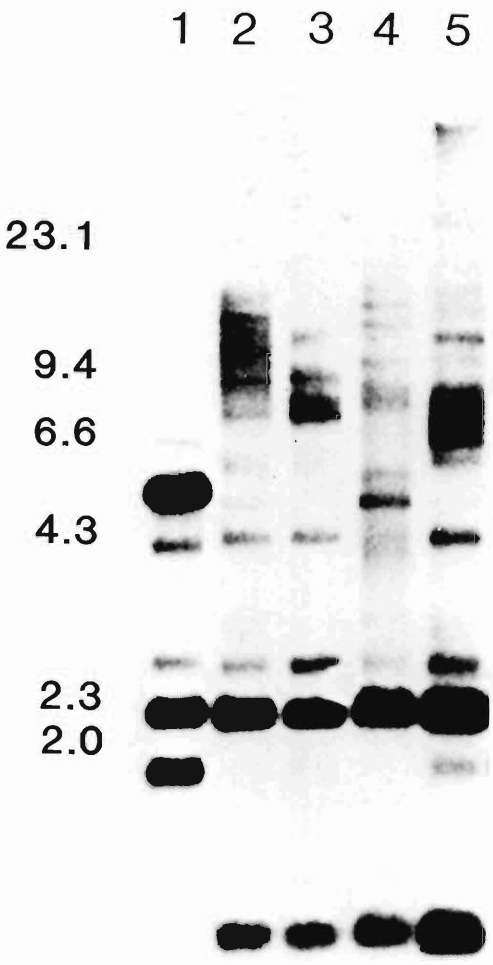

Figure 2. Southern blots of *T. spiralis* DNA screened with ^{32}P-labeled total RNA. DNA samples were digested to completion with Dra I, electrophoresed through a 0.8% agarose gel, and then blotted to a Nytran membrane filter. Blots were probed with [^{32}P]ATP-kinased *T. spiralis* RNA. Lane 1, Beltsville pig (*Sus scrofa*); 2, Montana wolf (*Canis lupus*); 3, grizzly bear (*Ursus arctos*); 4, polar bear (*Ursus maritimus*); 5, Pennsylvania fox (*Urocyon cinereoargenteus*).

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